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Hydrogen-Oxidizing Electron Transport Components in the Hyperthermophilic Archaebacterium *Pyrodictium brockii*

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The hyperthermophilic archaebacterium *Pyrodictium brockii* grows optimally at 105°C by a form of metabolism known as hydrogen-sulfur autotrophy, which is characterized by the oxidation of H_2 by S^0 to produce ATP and H_2S. UV-irradiated membranes were not able to carry out the hydrogen-dependent reduction of sulfur. However, the activity could be restored by the addition of ubiquinone Q_{10} or ubiquinone Q_6 to the UV-damaged membranes. A quinone with thin-layer chromatography migration properties similar to those of Q_6 was purified by thin-layer chromatography from membranes of *P. brockii*, but nuclear magnetic resonance analysis failed to confirm its identity as a ubiquinone. *P. brockii* quinone was capable of restoring hydrogen-dependent sulfur reduction to UV-irradiated membranes. Hydrogen-reduced-minus-air-oxidized absorption difference spectra on membranes revealed absorption peaks characteristic of *c*-type cytochromes. A *c*-type cytochrome with alpha, beta, and gamma peaks at 553, 522, and 421 nm, respectively, was solubilized from membranes with 0.5% Triton X-100. Pyridine ferrohemochrome spectra confirmed its identity as a *c*-type cytochrome, and heme staining of membranes loaded on sodium dodecyl sulfate gels revealed a single heme-containing component of 13 to 14 kDa. Studies with the ubiquinone analog 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide demonstrated that the *P. brockii* quinone is located on the substrate side of the electron transport chain with respect to the *c*-type cytochrome. These first characterizations of the strictly anaerobic, presumably primitive *P. brockii* electron transport chain suggest that the hydrogenase operates at a relatively high redox potential and that the H_2-oxidizing chain more closely resembles those of aerobic eubacterial H_2-oxidizing bacteria than those of the H_2-metabolizing systems of anaerobes or the hyperthermophile *Pyrococcus furiosus*.

The discovery of bacteria with optimal growth temperatures of 100°C or above has led to a major research effort to understand the biochemical basis for these extreme forms of thermophily. To date, most of the work has centered upon trying to elucidate and characterize the various metabolic components that allow bacteria to thrive at high temperatures. The two most intensively studied hyperthermophiles (defined by us [30, 31] as organisms that are able to grow at or above 100°C) are the heterotroph *Pyrococcus furiosus* (2, 5–7, 9–11) and the autotroph *Pyrodictium brockii* (25, 26, 28–32).

P. brockii was originally isolated from the solfatara fields off the coast of Volcano, Italy, by Stetter and his colleagues (35, 36). *P. brockii* has an optimal growth temperature of 105°C (36) and an absolute requirement for H_2 and CO_2, and it couples oxidation of H_2 to the reduction of sulfur to sulfide (26, 35, 36). This form of metabolism has been termed hydrogen-sulfur autotrophy (30, 36), a mode of growth that was originally recognized in hyperthermophilic archaebacteria. Even though CO_2 is utilized as the primary if not sole source of carbon in this form of metabolism, it should be noted that the growth of *P. brockii* is enhanced by the presence of yeast extract in the medium. One possible role of the yeast extract could be related to its ability to emulsify crystalline sulfur (25, 36); nevertheless, the presence of yeast extract does not relieve the requirement for either H_2 or CO_2 (26). The actual sulfur substrate reduced by *P. brockii* is probably polysulfide, the product of the nucleophilic attack of S-2 on S^0 rings (4).

No respiratory-type electron transport components of any hyperthermophilic bacterium have been identified. This is undoubtedly due to the inherent technical challenges in dealing with these unusual organisms. For example, yields of 4×10^7 to 5×10^7 *P. brockii* cells per ml are considered good (28, 30). We have identified a quinone and a *c*-type cytochrome in the membranes of *P. brockii*. The components were extracted from membranes and partially characterized. Furthermore, we present evidence that these components are involved in a sequential electron transport chain for the hydrogen-dependent reduction of sulfur to H_2S. The characteristics of this chain, involving a NiFeS hydrogenase functioning at a relatively high potential (29), quinone, and a *c*-type cytochrome, resemble those of aerobic H_2-oxidizing bacteria. Although our model may not be the complete electron transport chain, it is the minimum that is consistent with the available data and represents the first description of the electron transport chain of this bacterium.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were of reagent grade or better and were obtained from Sigma (St. Louis, Mo.), Alfa Chemical (Danvers, Mass.), or J. T. Baker Chemical (Phillipsburg, N.J.). High-purity solvents were obtained from Burdick and Jackson (Muskegon, Miss.). All gases were purchased from Linde Gases (Baltimore, Md.).

Bacterial strains and growth. *P. brockii* DSM 2708 was used in all experiments and was obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen, Germany). *P. brockii* was grown in bottles as previously described (31).

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Membrane preparation. *P. brockii* cells were harvested by first passing the cell culture through a Whatman no. 1 filter to remove large particles of crystalline sulfur. Cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. The pellet was washed four times in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid (pH 8.0) containing 100 mM NaCl. Cells from 1 liter of culture were resuspended in 4 ml of buffer. After the harvest, the cells were homogenized in an ice-cold ground glass homogenizer and then broken by two passages through a French press pressure cell at 20,000 lb/in^2 and 4°C. Unbroken cells and large particulate matter were removed by centrifugation at 10,000 × *g* for 20 min. Membranes were then pelleted by centrifugation at 110,000 × *g* for 1 h at 4°C. Membranes from 1 liter of cell culture were resuspended in a final volume of 1 ml of buffer and then homogenized in an ice-cold ground glass homogenizer.

UV irradiation and reconstitution. Membranes and ubiquinone were irradiated with UV light as previously described (40). Irradiation was carried out for 1 h at 4°C in a 1-ml quartz cuvette.

Experiments involving the reconstitution of hydrogen-dependent sulfide production in *P. brockii* membranes were carried out as follows. First 1.5 ml of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid (pH 8.0) buffer containing 100 mM NaCl and 100 μl of either irradiated or untreated membranes (containing between 20 and 50 μg of protein) were placed in a 12- by 75-mm glass tube. Then 0.03 g of elemental sulfur and 100 μl of either 100 μM bovine heart ubiquinone Q_{10}, 100 μM *Saccharomyces cerevisiae* ubiquinone Q_6, or *P. brockii* quinone in 100% ethanol was added to the tube. In control tubes, 100 μl of 100% ethanol was added. The tubes were tightly stoppered, placed in a heating block at 80°C, and sparged with 80% H_2–20% CO_2 for 5 min. The sample was then incubated at 80°C without sparging for an additional 55 min. The tube was cooled on ice and then assayed for the presence of hydrogen sulfide as described previously (8).

Experiments with 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were performed as described above, except that HQNO was added to a final concentration of 1 mM.

Quinone extraction. Quinone was extracted from *P. brockii* membranes by a modification of a previously described procedure (40). Total lipids were extracted from *P. brockii* membranes by the methods of Bligh and Dyer (3). The phospholipids were precipitated with cold acetone (18). The supernatant was dried under Ar, and the residue was resuspended in 0.2 ml of Ar-sparged chloroform-methanol (19:1).

Thin-layer chromatography (TLC) was performed with silica G plates (E.M. Merck) in petroleum ether-diethyl ether (85:15), and the plates were stained with I_2. The standards used in TLC were bovine heart ubiquinone Q_6 and *S. cerevisiae* ubiquinone Q_{10} (Sigma). The *P. brockii* quinone comigrated with ubiquinone Q_6. This spot was removed from the TLC and eluted into 100% ethanol for use in reconstitution experiments. The solvents used in the extraction procedure were of the highest quality available (Burdick and Jackson).

Cytochrome spectra. Sodium dithionite-reduced-minus-air-oxidized difference absorption spectra were determined for 1-ml fractions of membranes that had been solubilized in 0.5% Triton X-100. H_2-reduced-minus-air-oxidized difference absorption spectra were determined for 1-ml fractions containing between 0.6 and 1.0 mg of protein. Samples (1 ml) of intact membranes were made anaerobic by incubation at 37°C for 30 min with 20 μl (0.6 U) of Oxyrase (Oxyrase, Inc., Ashland, Ohio) and 20 μl of 0.5 M sodium succinate. The addition of succinate or oxyrase alone or in combination did not cause cytochrome reduction. The sample was then heated to 80°C, and the air-oxidized spectrum was recorded. The sample was reheated to 80°C, sparged with H_2 for 30 s, and then incubated at 80°C for 1 min without sparging. The sample was then scanned from 600 to 380 nm to obtain the H_2-reduced-minus-air-oxidized spectrum. Samples were scanned on a Perkin-Elmer model 557 spectrophotometer. Samples used in HQNO experiments were treated as described above, except that 20 μl of 50 mM HQNO (in ethanol) was added to the membrane sample and incubated at room temperature for 2 h before the addition of oxyrase. A control sample containing 20 μl of 100% ethanol was treated as above (see scan A of Fig. 3). All spectral analyses were performed with a reference cuvette that contained all components of the sample with the exception of the membranes. Pyridine ferrohemochrome spectral analysis on 0.5% Triton X-100-solubilized *P. brockii* membranes was done as described previously (27).

Hydrogenase assay. Hydrogen uptake activity was determined amperometrically (21, 37, 39) with the modifications described previously (31). All hydrogen uptake assays were done at 80°C.

SDS gel electrophoresis and heme stain. Membranes were suspended in 0.031 M Tris (pH 6.8)–0.04 M sodium dodecyl sulfate (SDS)–0.125 M NaCl–5% (vol/vol) glycerol–0.005% bromophenol blue and boiled for 5 min. After the SDS gels were loaded (see Fig. 4 legend), they were run as described by Laemmli (20). Heme staining of gels was done by the method of Francis and Becker (16).

RESULTS

Quinone identification. Isolated *P. brockii* membranes oxidize hydrogen when crystalline sulfur (S^0) is used as a terminal electron acceptor (30). The first indication that *P. brockii* utilized a quinone-dependent electron transport chain was discovered when membranes were irradiated with UV light. UV light is known to inactivate quinones (14, 40). H_2-dependent sulfide production was inhibited approximately 60% by the exposure of *P. brockii* membranes to UV light (Fig. 1A, lane B).

Quinones can be added and reconstituted into membranes after the quinone is dissolved in absolute ethanol. Adding the ethanolic quinone solution to an aqueous solution of membranes allows the hydrophobic quinone to insert into the membranes. Hydrogen-dependent sulfide production could be completely restored (in fact, enhanced) in membranes by the addition of 6 μM ubiquinone Q_{10} (Fig. 1A, lane C) or ubiquinone Q_6 (data not shown). When the known ubiquinones were exposed to UV light before they were added to the membranes, they did not restore hydrogen-dependent sulfide production in UV-irradiated membranes (Fig. 1A, lane D). UV irradiation did not affect the ability of the membrane-bound hydrogen uptake hydrogenase to oxidize hydrogen when 200 μM methylene blue was used as the electron acceptor (data not shown).

Quinone purification. Since ubiquinone Q_6 and ubiquinone Q_{10} were able to restore wild-type levels of sulfide production to membranes, these were used as standards for monitoring TLC purification of a *P. brockii* quinone. Total lipids were extracted from *P. brockii* membranes, and the phospholipids were removed by acetone precipitation. The remaining lipids contained an I_2-staining spot that comigrated

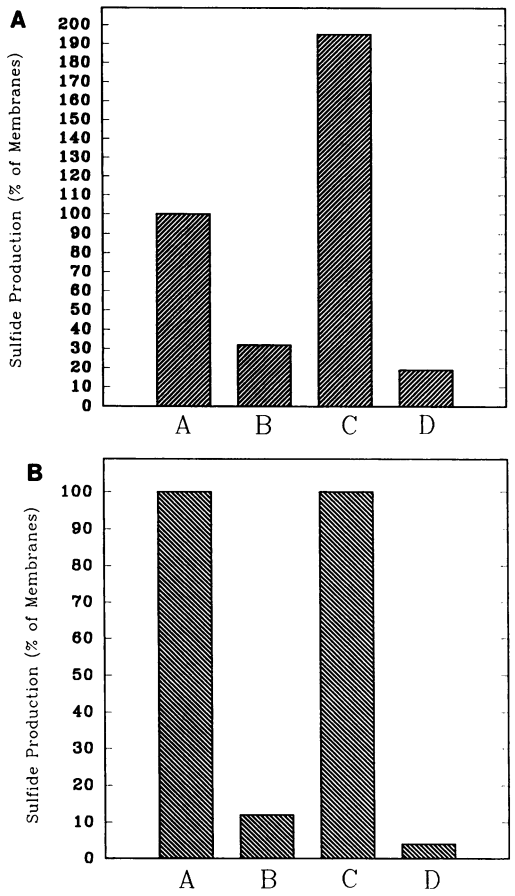

FIG. 1. (A) Restoration of hydrogen-dependent sulfide production by ubiquinone Q_{10}. Lanes: A, sulfide production by untreated membranes; B, sulfide production by membranes exposed to UV light for 1 h; C, reconstitution of sulfide production in UV-treated membranes by ubiquinone Q_{10} (added to UV-treated membranes as an ethanolic solution); D, ubiquinone Q_{10} exposed to UV radiation for 1 h before the UV-treated membranes were added. (B) Restoration of hydrogen-dependent sulfide production by purified *P. brockii* quinone. Lanes: A, untreated membranes; B, sulfide production by UV-irradiated membranes; C, reconstitution of sulfide production in UV-treated membranes by TLC-purified *P. brockii* quinone; D, UV-irradiated *P. brockii* quinone added to UV-treated membranes.

with ubiquinone Q_6 on TLC. This spot was the major spot visible on I_2-stained TLC plates. In our system, ubiquinone Q_6 had an R_f value of approximately 0.276 and the *P. brockii* quinone had an R_f value of 0.268. This spot was scraped off of the TLC plate and eluted into 100% ethanol. Subsequent reconstitution experiments similar to those of Fig. 1 showed that the purified *P. brockii* quinone was able to completely restore hydrogen-dependent sulfide production to UV-irradiated membranes (Fig. 1B, lane C). More than 100% activity relative to that of the wild-type nonirradiated membranes was not achieved by adding more *P. brockii* quinone (data not shown). When the ethanolic solution of purified *P. brockii* quinone was irradiated with UV light, it was unable to restore hydrogen-dependent sulfide production to UV-damaged membranes (Fig. 1B, lane D). These results correlate very strongly with those seen for the known ubiquinones Q_{10} and Q_6. It should be noted that the ability to restore hydrogen-dependent sulfide production varied between preparations of *P. brockii* quinone, but the results shown in Fig. 1B are representative of a typical reconstitution experiment. This variability is probably due to variations in the yield of quinone from the purification procedure. We were unable to quantitate the amount of quinone that was extracted from each preparation. Thus, we added constant volumes rather than quantities of purified *P. brockii* quinone to our reconstitution experiments. However, we were able to consistently restore significant levels of hydrogen-dependent sulfide production to UV-damaged membranes by adding purified *P. brockii* quinone. The restoration of hydrogen-dependent sulfide production in UV-damaged membranes by the purified *P. brockii* quinone strongly suggests that the spot that comigrates with ubiquinone Q_6 is the electron transport component that was damaged by UV light. However, when the nuclear magnetic resonance spectroscopy data of three separate preparations of the purified *P. brockii* quinone were analyzed (by J. Berg of Johns Hopkins University), we were unable to assign a specific structure to the quinone. Ready and routine determination of such spectra was confounded by the low cell yields characteristic of this bacterium (see above). Nevertheless, the spectra were consistent with a ring structure containing isoprenoid-like units; however, the ring structure data did not definitively match any of a number of quinones (34), including ubiquinones, menaquinones, plastoquinones, and even S-containing quinones from thermophiles (13, 17) (data not shown).

Cytochrome *c* identification and HQNO inhibition experiments. Difference absorption spectra (hydrogen reduced minus air oxidized) of membranes at 80°C revealed peaks characteristic of *c*-type cytochromes (Fig. 2A), with alpha and beta peaks at 554 and 528 nm, respectively. No peaks characteristic of other types of cytochromes were observed, and no peaks were obtained without the addition of H_2 (Fig. 2A). Therefore, no endogeneous reductant is present in the membrane preparation. A *c*-type cytochrome was solubilized from *P. brockii* membranes with 0.5% Triton X-100 (Fig. 2B). The Triton X-100-solubilized *c*-type cytochrome had clear alpha, beta, and gamma peaks at 553, 522, and 421 nm, respectively, when reduced with sodium dithionite (Fig. 2B). The use of Triton X-100 (at 0.5%) was sufficient to solubilize all of the cytochrome *c* from the membrane (data not shown). It is interesting to note that, in comparison, the solubilization of hydrogenase from *P. brockii* membranes required 2% Triton X-100 (29). When subjected to pyridine in basic conditions, *c*-type cytochromes give a characteristic peak at 550 nm in dithionite-reduced-minus-air-oxidized difference spectra (27). Such a pyridine ferrohemochrome spectrum of the detergent-solubilized *P. brockii* cytochrome is shown in Fig. 2C, with a sharp alpha peak at 550.5 nm.

To elucidate the order of the components in the electron transport chain, the quinone analog HQNO was used. HQNO has been found to block hydrogen-dependent electron transport in a variety of eubacteria, including *Bradyrhizobium japonicum* (22, 23) and *Azotobacter vinelandii* (40). In *P. brockii* membranes, HQNO was capable of strongly inhibiting hydrogen-dependent sulfide production by intact membranes when present at a concentration of 1 mM (Fig. 3, lane B). It also inhibited sulfur-dependent hydrogen oxidation, although not as strongly (Fig. 3, lane D). Therefore, HQNO was a suitable inhibitor for the study of the H_2-oxidizing chain at 80°C. Methylene blue-dependent hydrogen oxidation was not inhibited by HQNO; in fact, the rate of methylene blue-dependent hydrogen oxidation

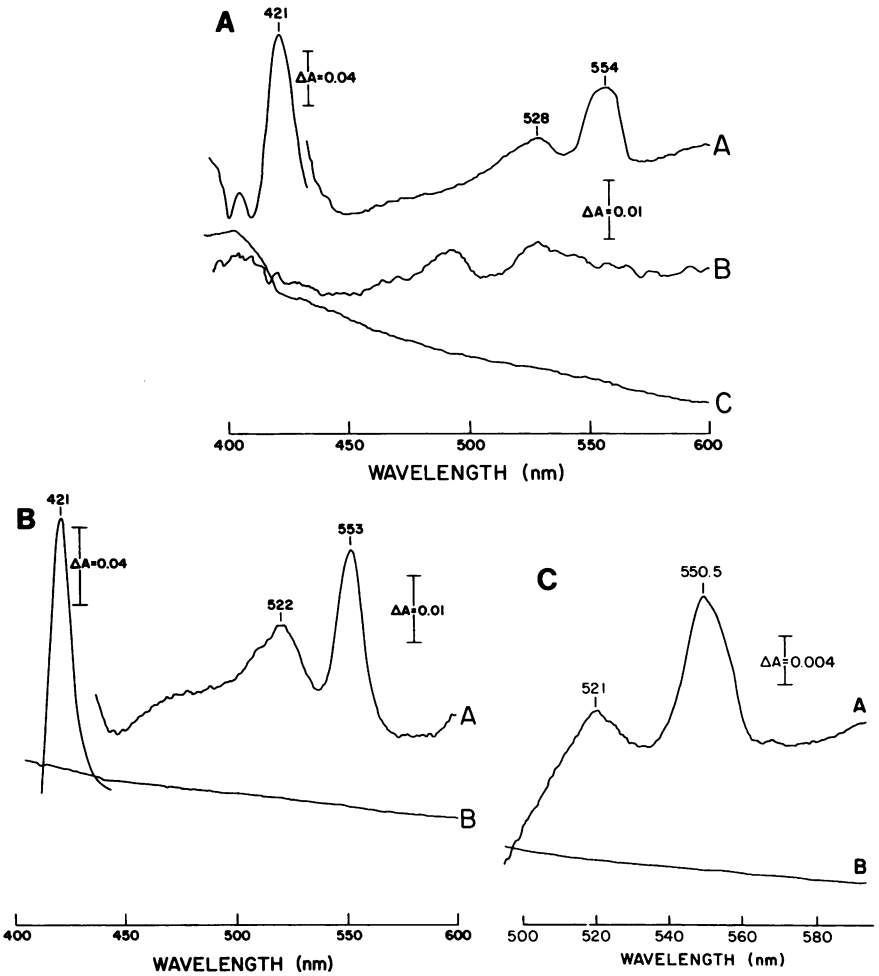

FIG. 2. (A) Inhibition of cytochrome reduction by HQNO. All samples were treated as stated in Materials and Methods. Curves: A, H_2-reduced-minus-air-oxidized spectrum of a membrane sample that received 20 μl of ethanol before the absorption spectrum measurement; B, as A, except the sample received 20 μl of 50 mM HQNO in ethanol; C, air-oxidized spectrum of a membrane sample. (B) Sodium dithionite-reduced-minus-air-oxidized difference absorption spectrum of *P. brockii* membranes solubilized in 0.5% Triton X-100. A 1-ml fraction of the supernatant (solubilized) fraction containing 0.8 mg of protein in a 10-nm-path-length glass cuvette was scanned from 600 to 380 nm. Curves: A, sodium dithionite-reduced-minus-air-oxidized spectrum; B, air-oxidized spectrum. (C) Pyridine ferrohemochrome difference absorption spectrum. Curves: A, sodium dithionite-reduced-minus-air-oxidized spectrum; B, air-oxidized spectrum. A 1-ml fraction of the supernatant (solubilized) fraction containing 1.5 mg of protein was scanned from 600 to 380 nm.

seemed to increase about twofold with the addition of HQNO.

When intact membranes from *P. brockii* were incubated with H_2 and HQNO and then used for difference spectroscopy, the spectral peaks from the previously seen *c*-type cytochrome were eliminated (Fig. 2A, scan B); therefore, HQNO inhibits cytochrome reduction. This result indicated that the *c*-type cytochrome is nearer the sulfur-reducing side and downstream of the HQNO block in the electron transport chain.

The *P. brockii* membranes were boiled in SDS and then subjected to SDS-polyacrylamide gel electrophoresis and heme staining to identify the size of the *c*-type cytochrome polypeptide. The gels (Fig. 4) revealed a single heme-staining component with an estimated molecular mass of about 13 to 14 kDa, slightly larger than the positive control (horse heart cytochrome *c*) with a molecular mass of 12.4 kDa.

Quinones are not autooxidizable, and therefore they can be extracted from membranes in a manner that preserves their redox state (19, 23). We determined the relative oxidation-reduction state of the quinone in membranes containing HQNO and H_2. This experiment was performed on membranes that had been incubated with H_2 in the presence of sulfur and with HQNO (Fig. 5A). Isolated *P. brockii* quinone showed spectral peaks at 290 and 247 nm (Fig. 5). These peaks are consistent with those seen for various types of bacterial quinones, including both ubiquinones and menaquinones (12, 19, 34). The spectra obtained (scanned from 400 to 190 nm) from this preparation did not change significantly at 290 or 247 nm upon the addition of the reductant sodium borohydride to the sample in the cuvette, indicating that the quinone was already in the reduced state (Fig. 5). Quinone extracted from membranes treated in the same manner but without the addition of HQNO showed an increase in absorption in these areas when sodium borohydride was added (Fig. 5B), suggesting that the quinone was not fully reduced. These results indicate that quinone reduction is

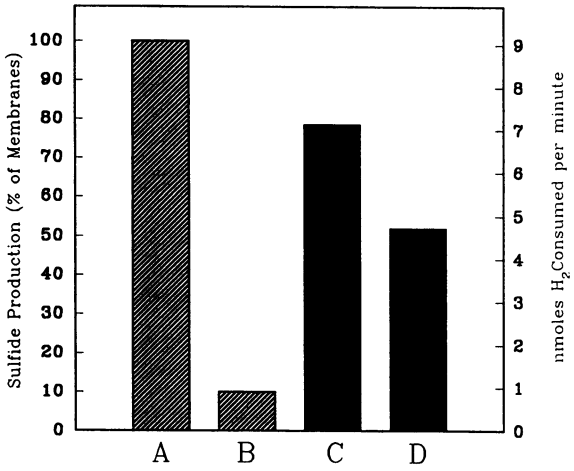

FIG. 3. Inhibition of sulfide production and hydrogen uptake by HQNO. Lanes: A, hydrogen-dependent sulfide production by membranes; B, hydrogen-dependent sulfide production by membranes in the presence of HQNO; C, sulfur-dependent hydrogen uptake by membranes; D, sulfur-dependent hydrogen uptake by membranes in the presence of HQNO.

located before the HQNO blocking site in the respiratory chain.

DISCUSSION

Although *P. brockii* is a strict anaerobe, its use of a quinone in the H_2-oxidizing chain is similar to the aerobic H_2-oxidizing electron transport chains in *B. japonicum* and *A. vinelandii* (23, 40) as well as in other aerobic H_2-utilizing bacteria (1). Whether the hyperthermophilic archaebacterial H_2-oxidizing chain utilizes menaquinone (present in some eubacterial anaerobes) or ubiquinone (common in eubacterial aerobes) or some other type of quinone must await further physical analyses of the *P. brockii* quinone. Thus far our physical analyses by nuclear magnetic resonance have not yielded definitive data on this. Previous investigations into the hydrogen uptake hydrogenase from *P. brockii* have shown that the hydrogenase enzyme has many similarities to NiFeS hydrogenases of aerobic eubacteria (29, 31). The

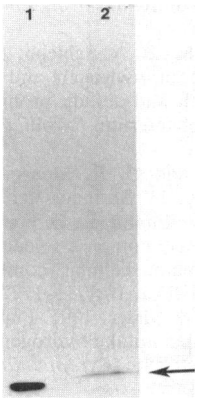

FIG. 4. Heme stain of cytochromes after SDS-polyacrylamide gel electrophoresis. Lanes: 1, horse heart cytochrome *c* (10 μg); 2 (arrow), *P. brockii* cytochrome *c* (84 μg of membrane protein was loaded).

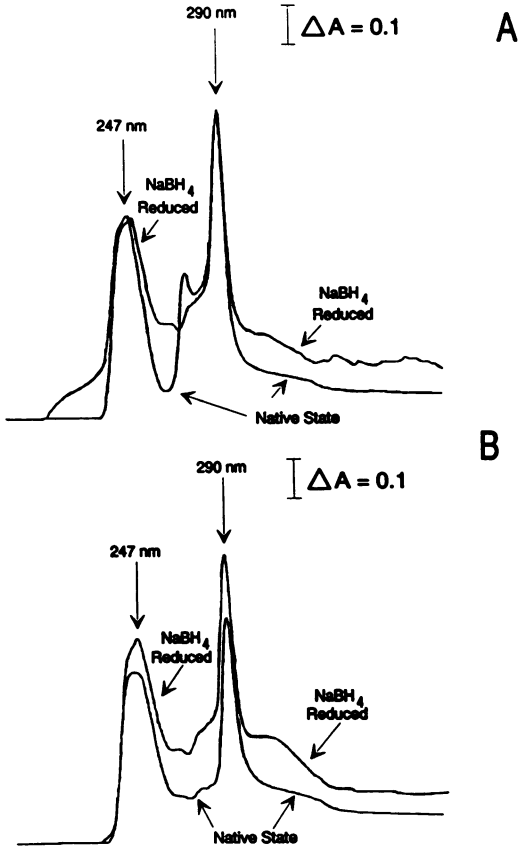

FIG. 5. Absorbance spectra of *P. brockii* quinone scanned from 400 to 190 nm. (A) Spectrum of *P. brockii* quinone extracted from membranes incubated with H_2, S^0, and HQNO. (B) Spectrum of *P. brockii* quinone extracted from membranes incubated with H_2 and S^0 only. Also shown are spectra from quinone in the originally extracted state (Native State) and after the addition of sodium borohydride to the cuvette ($NaBH_4$ Reduced).

presence of other similar components (quinone and cytochrome) in the *P. brockii* hydrogen-dependent electron transport chain serves to reinforce the idea that hydrogen oxidation and energy production in *P. brockii* at least resemble what has been seen in aerobic H_2-oxidizing eubacteria.

The presence of a *c*-type cytochrome is also interesting. Low-potential *c*-type cytochromes have been seen in some heterotrophic sulfur- and sulfate-reducing eubacteria. The presence of a *c*-type cytochrome in *P. brockii* may suggest that *c*-type cytochromes play a unique role in hydrogen-sulfur autotropy. In some *Desulfovibrio* strains, only cytochrome c_3, in addition to hydrogenase, is required to allow reduction of sulfate (15). This presents the possibility that the *P. brockii* cytochrome *c* also is part of or associated with the terminal S^0-reducing protein in electron transport. However, if it is the only cytochrome in the *P. brockii* membranes, it is probably a quinol oxidase as well. Such a function for a *c*-type cytochrome is unusual (1). Further studies will be needed to clarify this point. Our studies also suggest that the *c*-type cytochrome functions at a potential more positive than that of quinones, and thus the *c*-type cytochrome of *P. brockii* may not be similar to the low-potential types described, for example, in *Desulfovibrio* species. Interestingly, sulfur reduction by *Wolinella succi-*

nogenes with formate as the electron donor requires neither a quinone nor a cytochrome (33).

We propose the following model for electron transport in *P. brockii* as being the minimum that is consistent with our data. First, hydrogen is oxidized by the membrane-bound hydrogen uptake hydrogenase that we previously investigated. The electrons generated by this oxidation are then used to reduce a quinone, which in turn is oxidized by a *c*-type cytochrome. The membrane-bound character of the hydrogenase and the membrane association of the cytochrome may reflect the requirement of these proteins for interacting with the hydrophobic quinone. This model does not preclude the possibility of other components being present. For example, we have not yet identified the components of the terminal sulfur reductase. Although the *c*-type cytochrome may fill the sulfur reductase role, we have not attempted to assign this function to the *c*-type cytochrome. In addition, there may be other components, such as a ferredoxin or flavodoxin, that are involved with *P. brockii* hydrogen-oxidizing electron transport. It should be noted that the energy couple for H_2 to S^0 is only 144 mV (38). This limited energy potential might constrain the number of components permitted to efficiently capture energy in the electron transport chain.

We also do not propose a "sidedness" to the components of this electron transport chain. The production of scalar protons is important in the energy-generating metabolism of several H_2-metabolizing bacteria, particularly *Desulfovibrio* species (24); however, for *P. brockii* there are currently no data to suggest that production of scalar protons occurs.

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T.D.P. and L.K.B. contributed equally to this paper.

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